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Co-inoculated *Plasmopara viticola* genotypes compete for the infection of the host independently from the aggressiveness components

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Abstract During *Plasmopara viticola* epidemics only few genotypes produce most of the secondary lesions and dominate in the population. Selection of dominant genotypes is hypothesized to be linked to environmental conditions and can occur rapidly, particularly if there is also difference between genotypes in terms of fitness and aggressiveness. Measurements of aggressiveness components can largely determine the rate of epidemic development, although the components of aggressiveness do not take into account potential direct competition between genotypes. Differences in aggressiveness have been also reported to be greater under non-optimal conditions suggesting for genotype adaptation to different conditions. To evaluate differences in latency at non-optimal conditions, we characterized genotypes deriving from different climatic regions at three different temperatures (15, 25 and 35 °C) and we found no differences. To investigate whether other factors may impact on competition between *P. viticola* genotypes, we evaluated polycyclic infections of *P. viticola* by co-inoculating three genotypes with similar aggressiveness components in two different co-inoculation experiments and an increasing prevalence of one of the two genotypes was observed. Competition was not related to the origin of the genotype and we hypothesize that competitive selection is modulated by differences in the secretion of effector molecules which can contribute to the establishment of dominant genotypes over an epidemic season.

Keywords Fitness index · Competitive SSR amplification · Effectors · *Plasmopara viticola*

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Introduction

Downy mildew is one of the most damaging fungal diseases of grapevine (*Vitis vinifera*) worldwide. The causal agent, *Plasmopara viticola* (Berck. and Curt.) Berl. and de Toni, is a heterothallic (Wong et al. 2001) diploid oomycete and obligate biotrophic parasite native to North America. The pathogen overwinters as sexually-produced oospores in fallen leaves and the disease cycle starts when the oospores germinate producing sporangia and zoospores, which cause the primary infections (Gessler et al. 2011). All green parts of the plants which have active stomata can be attacked, and symptoms are easily recognized as yellowish lesions on leaves, known as "oilspots". When conditions are favourable, sporangia containing asexually-produced zoospores are released from the

primary lesion and asexual secondary infection cycles occur throughout the growing season alongside primary infections (Gessler et al. 2011).

Population genetic studies with microsatellite markers (SSR) have focused specifically on characterizing population structures in the field on large and fine spatial scales. These studies have shown that *P. viticola* has high evolutionary potential due to large population sizes, high genotypic diversity, moderate migration ability of asexual propagules, a mixed reproductive system and random mating (Gobbin et al. 2006). Primary oosporic infections have been shown to contribute in highly varying degrees to an epidemic throughout the season, and despite producing large amounts of sporangia (Reuveni 2003), only a few of these genotypes have been found to produce secondary lesions and to predominate in summer populations of the pathogen (Rumbou and Gessler 2004; Gobbin et al. 2005; Gobbin et al. 2006; Rumbou and Gessler 2006; Gobbin et al. 2007). The main reason the majority of genotypes fail to reproduce further after primary infection has been hypothesized as being linked to variable sensitivity to environmental conditions (Gobbin et al. 2005). Moreover, selection of dominant genotypes within populations can occur rapidly, particularly if there is a considerable difference between genotypes in terms of their fitness (Young et al. 2009), defined as the ability of a phenotype to contribute to the next generation (Antonovics and Alexander 1989). In the interaction between plant and pathogen genotypes, single generation measurements, such as infection efficacy, latent period, spore production rate, infectious period and lesion size are commonly used as "quantitative traits related to pathogenicity" which determine the aggressiveness of single isolates (Pariaud et al. 2009). Many studies have documented differences in aggressiveness among isolates belonging to the same pathotype (Pariaud et al. 2009) and, in experiments of co-inoculation of different *P. viticola* isolates, changes in their frequency may be largely dependent on differences in the aggressiveness components measured for the single isolates (Corio-Costet et al. 2011). However, while the aggressiveness of individuals may affect the competition between them and could explain the selection of dominant genotypes in field, it is also possible that direct competition or other mechanisms between genotypes also plays a role (Young et al. 2009). Among pathogen isolates, differences in aggressiveness have also been reported to be greater under non-optimal conditions (Pariaud et al. 2009), suggesting that differential responses in terms of aggressiveness may exist between genotypes originating from different climatic regions where genotypes may have been adapted to growing at different conditions.

To study differences in growth among *P. viticola* isolates under non-optimal conditions and to evaluate whether factors other than aggressiveness components could be responsible for genotype selection, we evaluated: i) differences in latency in *P. viticola* genotypes deriving from different climatic regions at non-optimal conditions; and ii) polycyclic infections of *P. viticola* in a competitive environment by co-inoculating genotypes with similar aggressiveness components, measured considering the latent period, infection frequency and spore production of pathogen's life cycle (Corio-Costet et al. 2011). Competition between the genotypes was assessed using a method based on a microsatellite marker developed for *P. viticola* (Gobbin et al. 2003; Delmotte et al. 2006). Moreover, the microsatellite-based quantification method (Naef et al. 2006; Reininger et al. 2011) was optimized for quick detection and quantification of *P. viticola* genotypes at different stages of the asexual infection cycle.

Materials and methods

Collection and propagation of *Plasmopara viticola* isolates

Entire leaves or fragments with single *P. viticola* lesions were collected. Each single lesion presumably represents a single *P. viticola* genotype (Gobbin et al. 2003). Vegetal material was placed separately, abaxial side up, on wet blotting paper in Petri dishes and incubated overnight in the dark at 25 °C to induce sporulation. Sporangia produced by the lesions were harvested separately in 4 ml of cold (4 °C) distilled water and the suspensions were used to inoculate *Vitis vinifera* cv. Pinot Noir cuttings (rootstock Kober 5BB) by spraying the abaxial leaf surface. Plants were incubated overnight in the dark for 12 h at 25 °C with 99–100 % RH, and then maintained at 25 °C, 60±10 % RH. Once oilspot symptoms appeared, sporulation was induced by placing the plants in the dark overnight at 25 °C and 99–100 % RH (Perazzolli et al. 2011). The amount of inoculum was increased over

four consecutive infection cycles. Once infected leaves reached 80–100 % disease severity (i.e. percentage of leaf area covered by sporulation), part of them were harvested, immediately frozen in liquid nitrogen and kept at −80 °C for DNA extraction, while the remaining leaves were kept at −20 °C for long term storage of the isolates (Laviola et al. 2006).

Effect of temperature on latency in *Plasmopara viticola* genotypes from different regions

Six strains were randomly isolated from single leaf lesions originating from different climatic regions in 2009: two isolates were from northern Italy (Lat: 45.918378, Long: 11.071558), one from central Italy (Lat: 44.486326, Long: 11.333106) two from southern Italy (Lat: 41.136262, Long: 16.871567) and one from Israel (Lat: 31.741015, Long: 35.182514). Sporangia from each lesion were collected and propagated as described above. Leaves from the fourth to the sixth node starting from the apical meristem of 10 week old plants grown in greenhouse were detached so that all leaves were of similar age and size. Leaves were randomly placed abaxial side up on moistened filter paper in Petri dishes. Nine leaves per strain were inoculated with six droplets of 20 μl of 5×10^4 sporangia ml^{-1} and incubated in greenhouse under the conditions described above. The day after inoculation, leaves were divided into three groups and incubated in the greenhouse under different temperature conditions: two groups were incubated at 15 °C and 25 °C, respectively, until sporulation, while the third group was incubated at 35 °C for 3 days, then at 25 °C until sporulation. Sporangia appearance was monitored daily through visual notation to estimate the latent period, defined as the time (days) from inoculation to first sporulation.

Microsatellite SSR analysis of *Plasmopara viticola* isolates

DNA was extracted from 100 mg of infected leaves or from 10 mg of freeze-dried inoculum suspensions using the DNeasy plant mini kit (Qiagen, Hilden, Germany). Samples were amplified using primers targeting the polymorphic *P. viticola* SSR loci ISAnew, BERnew (Gobbin et al. 2003; Matasci et al. 2010), Pv7, Pv13, Pv14, Pv17 (Delmotte et al. 2006), where each forward primer was labeled with the fluorophore VIC (Applied Biosystems, Foster City, CA). PCR reactions were carried out with a T-Professional Thermocycler (Biometra, Goettingen, GE) using the DreamTaq DNA Polymerase (Fermentas, St. Leon-Rot, Germany). PCR product was diluted 1:10 with sterile water, and 0.5 μl was mixed with 9.2 μl of HiDi formamide and 0.2 μl of GeneScan 500 LIZ size standard (Applied Biosystems). Samples were denatured for 2 min at 94 °C and cooled on ice. Fragments were separated on an ABI PRISM 3130 sequencer (Applied Biosystems) following the manufacturer's instructions, and analyzed using the Genemapper v 4.0 software (Applied Biosystems). PCR reactions and fragment analysis were performed in triplicate.

Aggressiveness components and fitness index

Three genotypes named BO, VOL 1 and VOL 2 were selected based on SSR profiles and analyzed for latent period, infection frequency and spore production using the methods described by Corio-Costet et al. (2011). All experiments were carried out as described above however using leaf disks with diameter of 2 cm instead of whole leaves. Three replicates (plates) were used for each genotype. Each disk was inoculated with three droplets (15 μl) of 2.5×10^3 sporangia ml^{-1} on the abaxial side and incubated in greenhouse at 25 ° C. Sporangia appearance was monitored daily through visual notation to estimate the latent period (LP), defined as the time (days) from inoculation to first sporulation. Sporangia production was calculated 7 days after the inoculum as the total number of sporangia produced per plate (N_t) divided by the 562 deposited sporangia (N_{t0}) (Corio-Costet et al. 2011). Sporulating spots of all leaf disks of each Petri dish were suspended in 20 μl of water and the number of sporangia per plate was determined using a haemocytometer. Infection frequency (IF) was calculated as the proportion of inoculated spots per plate (Corio-Costet et al. 2011) on which lesions had developed 7 days post-infection. The fitness index (F_I) was calculated for each isolate using the formula described by Corio-Costet et al. (2011) $F_I = \mathrm{Ln}\,(N_t/N_{t0} \times IF \times 1/LP)$. Experiments were repeated twice independently.

Co-inoculations with two different *Plasmopara viticola* genotypes

Fresh sporangia deriving from the three selected isolates were used to prepare inoculum suspensions. Two

co-inoculations experiments were performed combining the different genotypes in two different couples (BO and VOL 1; VOL 1 and VOL 2). Leaves were washed in cold (4 °C) distilled water and sporangia concentration was adjusted to 10^5 sporangia ml^{-1}. Inoculum suspensions were prepared by mixing the genotypes in varying proportions and used to inoculate *V. vinifera* cv. Pinot Noir cuttings at the conditions previously described. In the first co-inoculation experiment (BO and VOL 1), genotype BO was inoculated at 10, 30, 50, 70 and 80 % of the total infection while in the second co-inoculation experiment (VOL 1 and VOL 2) genotype VOL 2 was inoculated at 30, 50 and 80 % of the total infection. Each inoculum suspension was sprayed onto three leaves of single plants and they were considered as repetitions in the experiment. Leaves were selected as previously described and approximately 2 ml of inoculum suspension per leaf was sprayed. As controls, plants were inoculated separately with genotypes alone. Residual inoculum suspensions of first inoculum solution (around 10 ml) were freeze-dried and used for DNA extraction as described above. Plants were maintained in greenhouse in the conditions previously described until all leaves showed symptoms. Infected leaves were then detached, individually transferred onto wet blotting paper in Petri dishes, and incubated overnight in the dark to induce sporulation. Disease severity was assessed in control plants as percentage of abaxial leaf area covered by sporulation (Perazzolli et al. 2011). Sporangia from each leaf were harvested separately in 4 ml of cold distilled water and each sporangia solution was uniquely used to inoculate one single leaf on a new plant. The leaves after washing off the sporangia were immediately frozen in liquid nitrogen and kept at −80 °C for DNA extraction. The infection procedure was repeated in order to cover three consecutive asexual infection cycles.

Competitive microsatellite PCR and analysis of fluorescence data

The quantification method based on competitive microsatellite PCR relies on co-amplification of microsatellites of different sizes (Naef et al. 2006; Reininger et al. 2011). Competitive PCR with the SSR marker Pv14 (Delmotte et al. 2006) and fragment analysis was performed as described above on DNA mixtures of the genotypes BO (Pv14 allele size: 125) and VOL 1 (Pv14 allele size: 121) and on DNA mixtures of the genotypes VOL 1 (Pv14 allele size: 121) and VOL 2 (Pv14 allele size: 125). Total fluorescence per sample was calculated as the sum of the heights of each peak. BO and VOL 2 normalized fluorescence was calculated as its peak height divided by total fluorescence. Normalized fluorescence values were plotted against the known quantities of genotype DNA and a linear regression analysis was performed on data points.

Statistical analysis

Data were analysed using the Statistica 9 software (StatSoft, Tulsa, OK). Data with normal distribution (*K-S* test, $P>0.05$) were validated for variance homogeneity (Leven's test, $P>0.05$) and the analysis of variance (ANOVA) was performed using Fisher's test to detect significant differences between genotypes ($P<0.05$). In case data were not homogeneous according to *K-S* test (spore production and infection frequency data) data were transformed in LOG_{10} and analysed as described above.

Results

Effect of temperature on latency in *Plasmopara viticola* genotypes from different regions

To evaluate differences in genotypes development we compared the latency of six strains deriving from four climatic regions at three different incubation temperatures. Sporulation occurred in all strains incubated at 25 °C 5 days after inoculation, while those incubated at 15 °C and those kept at 35 °C displayed sporulation 7 days after inoculation, showing that growth rates at different temperatures were independent of the origin of the strains. No difference in latent period was observed among isolates.

Genotype selection and measurement of fitness index

Of the *P. viticola* genotypes collected, three were selected for the following characteristics: different allele size at locus Pv14 for at least one genotype (VOL 1, Pv14 allele size 121; BO and VOL 2, Pv14 allele size 125) and homozygosis for this specific SSR marker. Given that they were collected in Bologna (Emilia

Romagna, Lat: 45.918378, Long: 11.071558) and Volano (Trentino region, Lat: 44.486326, Long: 11.333106) they were named BO, VOL 1 and VOL 2. Sporangia production and infection frequency were found to be similar in all three genotypes (Fig. 1a and b), as was latent period (5 days after inoculation at 25 C°). The calculated fitness index (Fig. 1c), which associates the spore production, infection frequency and latency period for all three genotypes, were ranging from mean values of 2.1 (VOL 2), 2.2 (BO) and 2.5 (VOL 1), however without being significantly different according to Fisher's test ($P>0.05$).

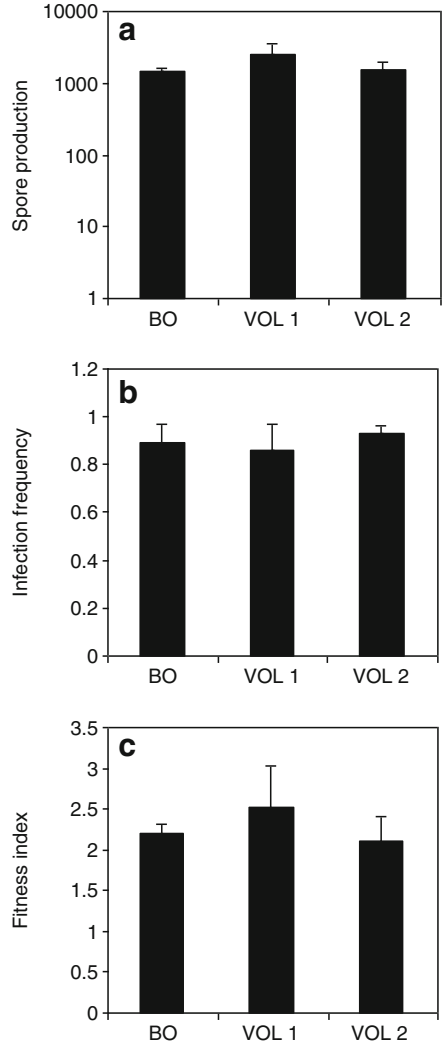

Fig. 1 Sporangia production (**a**), infection frequency (**b**) and fitness index (**c**) of *Plasmopara viticola* genotypes BO, VOL 1 and VOL 2. No differences were found between genotypes (Fisher's test, $P>0.05$). Mean values and standard errors of three replications of one representative experiment are presented

Optimization of competitive microsatellite PCR for identification and quantification of *Plasmopara viticola* genotypes

Amplification with the SSR Pv14 of the DNA of the two isolates BO (allele size 125) and VOL 1 (allele size 121) mixed in 1:1 ratio yielded two clearly distinct peaks with the same height (Fig. 2), showing that the two alleles were amplified in PCR with the same efficiency. We mixed DNA extracts of BO and VOL 1 in differing proportions and found a significant linear correlation ($R^2=0.9944$; $P<0.001$) between increasing BO concentration, corresponding to decreasing VOL 1 concentration, and the normalized fluorescence (Fig. 3). DNA extracts of VOL 1 (Pv14 allele size 121) and VOL 2 (Pv14 allele size 125) were also mixed in different proportions and for this allele couple we obtained similar results of significant correlation ($R^2=0.9860$; $P<0.001$) between the increasing VOL 2 concentration and the corresponding normalized fluorescence (data not shown). The light emission peaks not only provide information on the presence or absence of specific alleles, but their fluorescence intensity is in proportion to the amount of DNA in the original sample. The good correlation found allowed us to use the competitive microsatellite PCR with the SSR marker Pv14 to quantify two genotypes in a co-inoculation experiment. The contribution of each *P. viticola* genotype to infection was expressed as the fluorescence of its allele as a proportion of the total sample fluorescence.

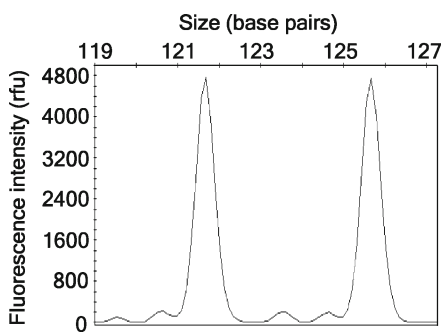

Fig. 2 Electropherogram from ABI GeneMapper software illustrating peak sizes (*base pairs*) and height (*fluorescence*) of the SSR marker Pv14 on the 1:1 DNA mixture of *Plasmopara viticola* genotypes BO (size 125.67; height 4,740) and VOL 1 (size 121.67; height 4,760). Both genotypes are homozygous for the SSR marker Pv14

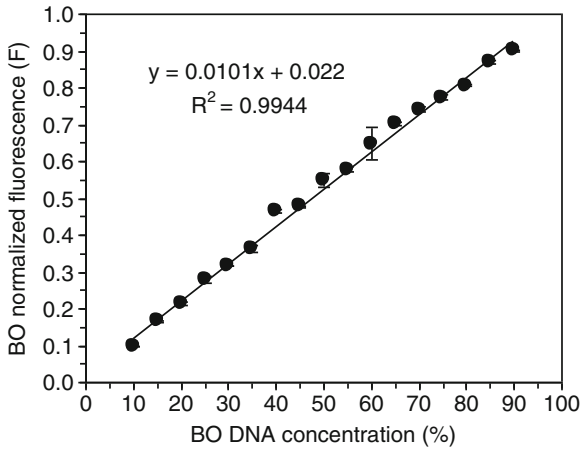

Fig. 3 Normalized fluorescence values and linear regression analysis for the SSR marker Pv14 of *Plasmopara viticola* DNA genotype BO (allele size 125) mixed with genotype VOL 1 (allele size 121) in proportions ranging from 10 to 90 % at intervals of 5 %. Mean values and standard error of three PCR replicates are reported

Co-inoculation dynamics of *Plasmopara viticola* genotypes

Competitive microsatellite PCR gave a precise estimation of the biomass of different *P. viticola* genotypes co-inoculated in the same plant tissue (Fig. 4). BO and VOL 1 genotypes were co-inoculated in five different starting proportions (Fig. 4a) and at each consecutive infection event (from inoculum to the third infection cycle) there was an increasing prevalence of genotype BO over genotype VOL 1. When the initial concentration of BO was greater than 50 %, it took two consecutive infection events for BO to predominate over VOL 1. When the initial concentration of BO was 53 and 36 %, BO prevailed at the third infection cycle, while at an initial concentration of 11 %, BO gradually prevailed over VOL 1 until the last infection cycle, although not completely (89 %).

In a second co-inoculation experiment (Fig. 4b), the VOL 1 was co-inoculated with a second isolate deriving from the same vineyard (VOL 2), and an increased prevalence of the genotype VOL 2 over the genotype VOL 1 was obtained at three different starting proportions. In the first two cycles, where VOL 2 concentration was 84 %, the initial proportions remained quite stable, however, at the third infection cycle, VOL 2 concentration reached a value higher than 98 %. When initial concentration was 53 % the VOL 2 concentration remains stable until the first cycle, then in the following cycles its frequency gradually increases to the value of 98 %, being practically the only genotype present in the infection. When VOL 2 was present at 31 % in the starting inoculum, gradually its concentration increases reaching the value of 91 % at the third infection cycle. Also in this co-inoculation experiment VOL 1 tends to disappear after repetitive infection cycles, permitting to VOL 2 to prevail. Control plants were inoculated with pure BO, VOL 1 and VOL 2 genotypes, resulting in successful infection and production of sporangia at every infection event. Moreover, analysis of the inoculum suspensions and infected leaves confirmed the absence of cross-contamination between genotypes during the experiment (data not shown).

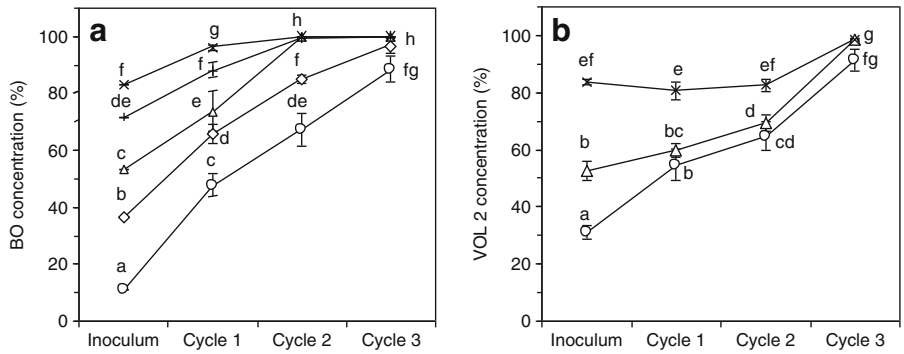

Fig. 4 Different proportions of *Plasmopara viticola* genotypes BO (**a**) and VOL 2 (**b**) expressed as percentages of total infections **a**: BO + VOL 1; **b**: VOL 1 + VOL 2) inoculated on grapevine leaves followed by three asexual infection cycles. Different starting proportions of the two genotypes were used in the two infection experiments. At each infection cycle, sporangia were harvested separately from each infected leaf and used to inoculate new single leaves. Each *point* represents mean and standard error of three infected leaves of the same plant. *Different letters* indicate significant differences according to a Fisher's test ($P<0.05$)

Discussion

In a given *P. viticola* population, particular genotypes dominate while others disappear and never produce secondary lesions (Gobbin et al. 2005). This could be attributed to differences in aggressiveness components, such as sporulation, duration of latency (Pariaud et al. 2009; Corio-Costet et al. 2011) or could be due to external factors such as resistance to applied fungicides or differences in microclimate conditions (Gessler et al. 2011). We initially tested the genotypes collected in different geographical locations (northern, central and southern Italy, and Israel) to ascertain whether different strains had adapted to grow at different temperatures and we were able to exclude the temperature as a factor affecting the growth (latency) of the isolates. Apart from external factors potentially influencing growth and self-reproduction, other factors such as competition between genotypes during asexual cycles may contribute to the dominance of a given genotype, indicating that additional, as yet identified, processes are playing a role. We therefore studied the dynamics of co-inoculations by *P. viticola* genotypes with similar aggressiveness components in order to assess potential competition during infection. To have a precise estimation of genotypes aggressiveness we referred to the measure of three quantitative traits of pathogen's life cycle such as infection efficiency, latent period and sporulation rate (Pariaud et al. 2009). From these different parameters, a composite fitness index (Corio-Costet et al. 2011) was calculated for each genotype, giving, as a result, that the three genotypes used in our co-inoculations experiments had similar aggressiveness; therefore, theoretically, the same chances to successfully produce an infection, grow within the plant tissue, and produce sporangia. To quantify the biomasses of the genotypes co-inoculated in the host, we developed a precise method based on competitive microsatellite PCR. Competitive PCR is a precise quantification tool (Zentilin and Giacca 2007) and, given the natural length polymorphism of the SSR marker Pv14, differently-sized microsatellite DNA deriving from different *P. viticola* genotypes can serve as mutual competitors. The method allowed us to differentiate and quantify the biomasses of different *P. viticola* genotypes within the same plant tissue with sufficient resolution to study their variation over time. This quantification method requires careful selection of microsatellite markers as they often generate stutter peaks, which can mask or overlap onto other alleles, and shorter alleles can be preferentially amplified (Daniels et al. 1998). These drawbacks were circumvented in our PCR assay by selecting genotypes presenting similar, but still clearly distinguishable, fragment lengths in homozygosis for the SSR marker Pv14, which were also suitable (sharp peak and low stutter bands) for the quantification method based on competitive PCR. Unlike Naef et al. (2006) and Reininger et al. (2011) we directly quantified the biomasses of the genotypes in the experimental samples without interpolation on the calibration curve. The fluorescence values obtained with the electropherogram were internally normalized and comparison with a standard was not necessary, on the assumption that the sum of the two peaks is equal to 100 and fluorescence of each peak is expressed as a percentage.

When *P. viticola* genotypes were individually inoculated, infections and subsequent sporulation occurred throughout the experiments at comparable rates. However, in both co-inoculation experiments, the two genotypes strongly compete, indicating the existence of other factors for genotype selection. Analysis of three consecutive co-inoculation cycles in two different co-inoculation experiments revealed a decreased frequency of the VOL 1 genotype in the infections, independently of the proportions in the initial inoculum. Whereas the tested genotypes had comparable aggressiveness components and fitness indexes under individual inoculations, VOL 1 had the part of the weaker competitor in the co-inoculation experiments, indicating the additional factors that may play a key role in competition. Although the mechanisms by which different genotypes compete are not well understood, for other oomycetes (i.e. *Phytophthora infestans*) it has been hypothesized that some genotypes could induce production of defence proteins in the host plant earlier than others in a competitive situation (Young et al. 2009). Genotypes that are able to overcome the earlier plant defence reaction would have a competitive advantage, which, coupled with subsequent inhibition of other genotypes, would explain competitive selection (Young et al. 2009). In the pathogenic process caused by *Peronosporaceae*, effector proteins are responsible for modulation of plant cell defence and/or induction of cell death thus creating a favourable environment for infection (Stassen and Van den Ackerveken 2011).

P. viticola does seem to modulate host cell defences through apoplastic and cytoplastic effectors secreted during the first stages of infection (Dìez-Navajas et al. 2008) and a first identification of effector genes have been recently described in germinating zoospores (Mestre et al. 2012). The genetic variability of effectors across isolates has been demonstrated in various oomycete species (Halterman et al. 2010) and in *P. infestans* isolates genetic variability at one effector locus has also been correlated with pathogen aggressiveness (Halterman et al. 2010). Thus, as hypothesized for *P. infestans* genotypes (Young et al. 2009), the more competitive *P. viticola* genotypes could have a more complex arsenal of effectors enabling it to suppress or overcome host resistance, while the less competitive genotypes are more vulnerable to the host defence mechanism triggered by the more competitive genotype. The results and interpretations presented here represent a valuable addition to current knowledge of the biology of this grapevine pathogen. Plant-pathogen interactions seem to be more complex than mere colonization of plant tissue, since different *P. viticola* genotypes react differently when they co-exist on a unique substrate. Thus, differences in the production of effector molecules could contribute to the establishment of dominant genotypes in the field over the course of an epidemic season of downy mildew.

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